State of the Art

HMGB1 as a Late Mediator of Lethal Systemic Inflammation

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CONTENTS

Gram-negative bacterial infection and systemic inflammation are widespread problems in critically ill patients. Administration of endotoxin (lipopolysaccharide, LPS), a product of gram-negative bacteria, to animals provides a model to study the biological cytokine cascade that is central to the pathophysiology of lethal systemic inflammation. Tumor necrosis factor (TNF) mediates the early cytokine response to lethal endotoxemia, and anti-TNF antibodies have been developed for clinical use in rheumatoid arthritis and Crohn's disease. High-mobility group 1 (HMGB1), a protein previously known only as a nuclear transcription factor, is now implicated as a mediator of delayed endotoxin lethality and systemic inflammation. Here we review the studies that led to the discovery of HMGB1 as a late mediator of systemic inflammation.

PROINFLAMMATORY RESPONSE IN SEPSIS

The balance of pro- and antiinflammatory mediators derived from the innate immune system defines the progression and severity of endotoxemia. If unbalanced, an overproduction of endogenous proinflammatory mediators, including cytokines, platelet-activating factor, oxygen radicals, and nitric oxide, synergistically interact to mediate hypotension, multiple organ failure, and death. Progression from sepsis to septic shock coincides with an increase in circulating levels of proinflammatory cytokines such as TNF, interferon γ (IFN-γ), interleukin 1β (IL-1β), and IL-6 (1). Studies of animal models suggest that inhibition of these early mediators suppresses particular facets of the pathological response. For instance, neutralizing antibodies to TNF, the first cytokine elaborated in the septic inflammatory cascade, prevent death in baboons when administered before or concurrent with lethal doses of live *Escherichia coli* (2). TNF is a necessary and sufficient mediator of septic shock in experimental animal models. First, TNF is produced in animals during septic shock (3, 4). Second, removing TNF from diseased animals, by either pharmacological strategies or genetic disruption, significantly improves survival after endotoxin challenge (2, 5, 6). Last, administration of TNF to normal animals reproduces the pathological sequelae of septic shock (7, 8). The identification of TNF as an essential mediator of gram-negative septic shock focused attention on the development of therapies directed at endogenous toxins. A number of other proinflammatory cytokines have since been implicated in the mediation of endotoxin lethality. For instance, IL-1 (9, 10), leukemia inhibitory factor (LIF) (11, 12), IFN-γ (13, 14), and migration inhibitory factor (MIF) (15–17) may each contribute to the pathogenesis of endotoxemia or septic shock. Clearly, the pathogenesis of sepsis is modulated by an interaction between these and perhaps other mediators. Each of these secondary mediators can be induced by TNF, and a detailed discussion of individual cytokine activities is beyond the scope of this review.

ANTIINFLAMMATORY RESPONSE IN SEPSIS

The early systemic release of TNF during endotoxemia activates lethal downstream pathological responses (7). Endotoxin also activates "antiinflammatory" mechanisms that counterregulate or suppress potentially injurious proinflammatory mediators. In LPS-stimulated monocytes, for example, the local accumulation of prostaglandin E_2 (PGE_2) inhibits the synthesis of proinflammatory cytokines that can restrain an acute cytokine response (18). Another local feedback mechanism is through spermine, a ubiquitous biogenic molecule that accumulates at sites of infection or injury, and posttranscriptionally inhibits the release of multiple proinflammatory cytokines (e.g., TNF, IL-1β, macrophage inflammatory protein 1α [MIP-1α], and MIP-1β) from macrophages and monocytes (19–22). Antiinflammatory cytokines (e.g., IL-10 and transforming growth factor β [TGF-β]) can participate in the downregulation of the inflammatory response. For instance, IL-10 can deactivate macrophages, and TNF levels in trauma patients are higher when IL-10 levels are depressed, a scenario that has been implicated in the onset of septic complications (23–25). TGF-β is a potent inhibitor of monocyte activation (26), and significantly elevated levels of TGF-β have been observed in monocytes derived from immuno-

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suppressed trauma patients (27). New evidence suggests that the central nervous system can directly, and rapidly, attenuate the TNF response to endotoxin through efferent vagus nerve signals to tissue-resident macrophages (28). This effect is mediated by acetylcholine, the principal neurotransmitter of the vagus nerve, which signals via nicotinic cholinergic receptors present on macrophages (28). Thus, the complex cytokine milieu in septic patients is characterized by the interaction between anti-inflammatory responses and potentially injurious proinflammatory responses that are tightly regulated by neural and humoral pathways.

THERAPEUTIC CONTROL OF SEPSIS

Several therapeutic agents that target TNF have been tested in clinical trials of sepsis, and a significant survival advantage has not been observed. One confounding factor is that TNF levels are not usually increased in enrolled patients, in part because of the rapid kinetics of the TNF response (29, 30). Serum TNF and IL-1β reach toxic levels in mice and human volunteers within 1–2 h after LPS infusion (3, 31), but delayed treatment with anti-TNF or anti-IL-1β fails to prevent late endotoxin deaths (2, 32). Paradoxically, endotoxin-responsive mice treated with lethal doses of endotoxin often succumb at latencies of up to 5 d, long after serum TNF and IL-1β have returned to basal levels (Figure 1). Transgenic mice rendered devoid of TNF receptors, although partially protected from endotoxin lethality, die several days after high-dose LPS administration (33, 34). On the basis of these and other observations, we reasoned that treatment strategies might be developed to target late-acting, clinically accessible mediators that are distinct from TNF. The combined insights gained from the failure of clinical trials of anti-TNF strategies (35, 36), as well as the observations surrounding the kinetics of TNF release as compared with the onset of lethality (3, 31), prompted a search for a late mediator of endotoxin-induced death.

WIDENING THE THERAPEUTIC WINDOW FOR SEPSIS

In an effort to broaden the therapeutic window for sepsis, we initiated a search for another macrophage-derived, putative mediator released relatively late after the onset of endotoxemia. We stimulated murine macrophage-like RAW 264.7 cells with LPS and screened the conditioned medium for proteins appearing after 16 h. LPS induced the appearance of a 30-kDa protein in the conditioned medium that was not apparent at earlier time points. The N-terminal amino acid sequence of the 30-kDa protein (i.e., G-K-G-D-P-K-K-P-R-G-K-M-S-S) was identical to murine HMGB1, a member of the high-mobility group 1 nonhistone chromosomal protein family (37, 38).

Nuclear HMGB1 as a DNA-binding Protein

Approximately 30 yr ago, HMGB1 was first copurified from nuclei with histones, and termed "high-mobility group" (HMG) protein because of its rapid mobility on electrophoresis gels (39). As a highly conserved protein, HMGB1 shares 100% identity in amino acid sequence between mouse and rat, and a 99% amino acid identity between rodent and human (37, 38, 40). HMG-1 has been renamed HMGB1 by a nomenclature committee (41). As a nonhistone chromosomal protein, HMGB1 has been implicated in diverse cellular functions, including determination of nucleosomal structure and stability, and binding of transcription factors to their cognate DNA sequences (42). Highly charged HMGB1 contains a continuous stretch of negatively charged (aspartic and glutamic acid) residues in the C terminus, and two internal repeats of positively charged domains ("HMG boxes") in the N terminus (Figure 2) (43). The

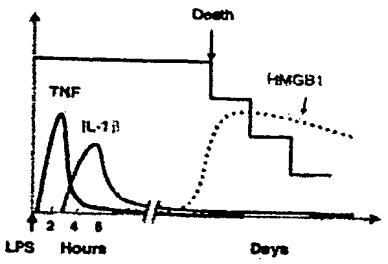

Figure 1. Early versus late mediators of endotoxin lethality. Vertebrates treated with lethal doses of LPS succumb at latencies of up to several days, long after serum TNF and IL-1β have returned to basal levels. The kinetics of HMGB1 release is delayed, and parallels the onset of lethality.

HMG boxes provide the structure-specific binding sites for DNA with secondary structures such as supercoiled DNA (44), cruciform DNA (45), four-way junctions (46, 47), and cisplatin-damaged DNA (48). These observations suggested a possible role for HMGB1 in DNA recombination, repair, replication, and gene transcription (42). Although HMGB1 has not been associated with gene transcription *in vivo* (49), it can stimulate transcription *in vitro* (50–52). HMGB1 bends DNA and facilitates binding of various transcription factors to their cognate sequences, including the steroid/nuclear hormones progesterone (53) and estrogen (54, 55), HOX proteins (50), and transcription factor II B (56). *Hmg1*-deficient mice are viable only for a few hours after birth, although parenterally administered glucose can prolong survival for a few days (57). The lack of chromosomal HMGB1 protein does not disrupt cell growth, but may affect the transcriptional regulation of certain genes, such as those activated by the glucocorticoid receptor (57).

Membrane HMGB1 as a Ligand for RAGE Receptor

HMGB1 localizes to the nucleus and cytoplasm of most cells, to the cell membrane of neuronal (neuroblastoma) cells (58), and to the filopodia of the advancing plasma membrane of neurites (58, 59), where it colocalizes and interacts with tissue plasminogen activator (t-PA). Membrane HMGB1 has been termed "amphoterin" because it has marked dipolar charge properties (Figure 2) (58). Local acceleration of t-PA-catalyzed plasminogen activation by HMGB1 at the leading edge of the cytoplasmic membrane has been implicated in cell migration (60), differentiation, and regeneration (61–63). HMGB1 can bind to the receptor for advanced glycation end products (RAGE) in a dose-dependent manner (63). The interaction between HMGB1 and RAGE can be competitively inhibited by addition of soluble RAGE or anti-RAGE IgG (63). RAGE is expressed on mononuclear phagocytes and vascular smooth muscle cells (62, 64, 65), and engagement of RAGE with ligands (such as advanced glycation end products [AGE], or HMGB1), activates not only the NF-κB signaling pathway (64, 66), but also the mitogen-activated protein kinase (MAPK) pathways (67).

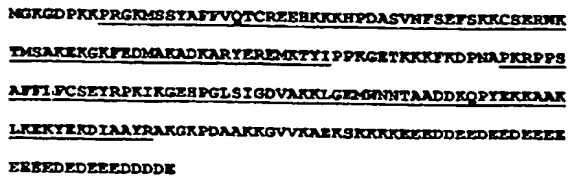

Figure 2. Amino acid sequence of murine HMGB1. The C-terminal portion of HMGB1 contains a continuous sequence between 35 and 40 aspartic and glutamic acid residues, and the N-terminal portion of HMGB1 comprises two internal repeats of a positively charged domain of about 80 amino acids (termed "HMG boxes") (shown by *underlines*).

Accumulation of Serum HMGB1 during Endotoxemia

To study the biology of HMGB1 as a mediator of systemic inflammation, recombinant HMGB1 (rHMGB1) was expressed in *E. coli*, purified to homogeneity, and used to generate polyclonal anti-HMGB1 antibodies for developing a quantitative immunoassay (68). Large amounts of HMGB1 are released by macrophage cultures in a time-dependent manner, with significant HMGB1 accumulation first detectable after 8 h of LPS stimulation (68). HMGB1 release by LPS-stimulated macrophages was not due to cell death, as judged by trypan blue exclusion and lactate dehydrogenase (LDH) release. The inducible nature of HMGB1 release has been observed in murine primary peritoneal macrophages, human primary peripheral blood mononuclear cells (huPBMCs) (68), as well as murine pituitary cells after stimulation with proinflammatory cytokines (e.g., TNF and IL-1β) (69). Notably, TNF- and IL-1β-mediated inflammatory effects are often highly synergistic. For instance, TNF and IL-1β have been shown to interact synergistically in inducing polymorphonuclear leukocyte migration (70), and the production of cytokines (e.g., IL-6) (71) and prostaglandin E_2 (PGE_2) (72). It remains to be investigated whether such a synergistic interaction between TNF and IL-1β plays a role in the regulation of HMGB1 release during systemic inflammation.

Systemic HMGB1 accumulation during endotoxemia was measured in the serum of mice after LPS administration. Serum HMGB1 was minimally detectable by 8 h after administration of a median lethal dose (LD_{50}) of LPS, and increased to a prolonged plateau level from 16 to 32 h after LPS treatment. This delayed release distinguishes HMGB1 from previously described early cytokine mediators of LPS lethality. In view of the late and prolonged kinetics of HMGB1 in mice, we thought that it might be possible to detect increased serum HMGB1 levels in critically ill patients, because clinical signs of sepsis typically develop long after the early cytokine response to the acute infection. We probed for immunoreactive material in the serum of 8 healthy normal subjects and 25 critically ill patients with surgical sepsis and sepsis-induced organ dysfunction (i.e., hypotension, lactic acidosis, disseminated intravascular coagulation, hypoxemia, or decreased urine output) secondary to infection. Serum HMGB1 was not detectable in serum of normal subjects, but was significantly increased in all critically ill patients with sepsis (68). Moreover, serum HMGB1 levels were significantly higher in septic patients who did not survive as compared with survivors (68).

Hemorrhagic shock, like septic shock, is characterized by activation of a cytokine cascade, even in the absence of an initial stimulation by bacterial products. As with septic shock, TNF is released during hemorrhagic shock (73), and plays an important role in the pathophysiology of the disease (74). Because TNF can induce the release of HMGB1 from macrophage-monocyte (68) and pituicyte cultures (69), it is possible that hemorrhagic shock itself, in the absence of infection, can stimulate the systemic release of HMGB1. Indeed, in a clinical case, serum HMGB1 levels increased significantly within 24 h after the onset of hemorrhagic shock, and returned toward basal levels as the clinical condition improved (75). The role of other inflammatory stimuli in activating HMGB1 release in the absence of infection is unclear.

Anti-HMGB1 Antibodies Protect against Lethal Endotoxemia

The biological role of extracellular HMGB1 was studied *in vivo* by passive immunization of unanesthetized endotoxemic mice. A single dose of anti-HMGB1 antiserum administered 30 min before an LD_{100} dose of LPS failed to prevent LPS-induced death. The delayed kinetics of HMGB1 accumulation in serum suggested to us that effective neutralization of a late-appearing mediator might require delayed dosing with antibodies. By treating animals with three doses of antiserum (30 min before LPS, and 12 and 36 h after LPS), we observed a significant improvement in survival, with 70% of treated animals surviving as compared with 0% survival of controls treated with three matched doses of preimmune serum. Delaying the first dose of anti-HMGB1 antibodies for at least 2 h after LPS still conferred significant protection against an LD_{100} dose of LPS (68). More recent data (H. Yang and K. J. Tracey, unpublished) indicate that anti-HMGB1 antibodies confer significant protection against sepsis in an animal model of cecal perforation, even when antibody administration is delayed by 24 h. This suggests that it may now be possible to develop inhibitors of HMGB1 for treatment of systemic inflammation, and that the therapeutic window for these therapies may be significantly wider than for TNF-targeted interventions.

Anti-HMGB1 Antibodies Protect against LPS-Induced Acute Lung Injury

Systemic inflammation is frequently complicated by lung injury. In a widely used animal model of LPS-induced acute lung injury, administration of anti-HMGB1 either before or after endotoxin treatment significantly decreased endotoxin-induced neutrophil accumulation into the lungs, and attenuated the severity of lung edema produced by intratracheal administration of endotoxin (76). Despite the ameliorative effects of anti-HMGB1 antibodies on the development of lung injury and neutrophil accumulation, this treatment had no effect on endotoxin-induced increases in pulmonary concentrations of IL-1β, TNF, or MIP-2, indicating that endogenous HMGB1 is a mediator of acute lung injury. The role of HMGB1 in the pathogenesis of acute lung injury appears to be distinct from the effect on early-acting proinflammatory cytokines. To examine the toxicity of HMGB1 itself, a highly purified preparation of HMGB1 was administered intratracheally to LPS-resistant C3H/HeJ mice. Lung neutrophil accumulation and edema increased in a dose-dependent manner at 8 and 24 h after HMGB1 administration. Intratracheal administration of HMGB1 led to significant increases in tissue levels of proinflammatory cytokines, including IL-1β, TNF, and MIP-2. Histological examination of tissue sections prepared from the lungs of animals treated for 24 h with pathophysiological doses of HMGB1 revealed evidence of an acute diffuse inflammatory response, with accumulation of neutrophils in the interstitial and intraalveolar areas, interstitial edema, and protein exudation into the alveolar space (76). These pathological changes are typically observed in response to acute lung injury mediated by endotoxin, TNF, and other proinflammatory stimuli. However, these effects could not be attributed to trace amounts of endotoxin coadministered with the HMGB1, because C3H/HeJ mice do not respond to low doses of endotoxin. It is reasonable to consider that HMGB1 may contribute to the pathogenesis of acute lung injury and systemic inflammation.

HMGB1 as a Macrophage-activating Cytokine

The data reviewed above were the first to implicate HMGB1 as a cytokine. To further examine the cytokine activity of HMGB1, HMGB1 was added to human primary blood mononuclear cell cultures. HMGB1 significantly increased the release of TNF in a dose-dependent manner (77). Upregulation of TNF mRNA after treatment with HMGB1 was observed within 4 h after addition of rHMGB1, but peak TNF mRNA levels were not achieved for 10 h. This stimulation was not attributable to small quantities of LPS that may have contaminated the rHMGB1, for several reasons. First, the kinetics for HMGB1- and LPS-induced TNF synthesis are distinctly dif-

TABLE 1. PATHOPHYSIOLOGY OF HMGB1 RELEASE

	Cell Culture	Mice	Humans
Release of HMGB1	Increased levels in pituicyte cultures 8 to 10 h after TNF stimulation,* and in macrophage cultures 8 h after LPS stimulation†	Increased in serum 16 h after LPS administration, and remains elevated for > 36 h†	Elevated levels of HMGB1 detectable in serum of patients with surgical sepsis† and hemorrhagic shock‡
Neutralization of HMGB1 by anti-HMGB1 antibodies	Not known	Protects against LPS-induced acute lung injury,§ and lethality†	Not known
Administration of recombinant HMGB1	Stimulates release of TNF and other cytokines in cultures of macrophage-monocytes‖	Causes acute lung injury,§ and lethality†	Not known

* See Wang *et al.* (69).
† See Wang *et al.* (68).
‡ See Ombrellino *et al.* (75).
§ See Abraham *et al.* (76).
‖ See Andersson *et al.* (77).

ferent, with the late peak for HMGB1-stimulated TNF synthesis (8–10 h after stimulation) being incompatible with the rapid kinetics for LPS-induced TNF synthesis (peaks within 2–3 h). Second, coaddition of LPS-neutralizing agents such as polymyxin B in amounts that exceeded by 100-fold the concentration necessary to neutralize contaminating LPS (as revealed by the *Limulus* amebocyte lysate [LAL] endotoxin assay) fails to inhibit HMGB1-induced cytokine synthesis. Third, proteolytic degradation of HMGB1 by trypsin abolishes the activity of HMGB1 as a cytokine stimulus, whereas treatment of LPS with trypsin does not affect the activity of LPS in stimulating TNF release (77). Pathological doses of HMGB1 amplify the cytokine cascade during systemic inflammation; however, the contribution of this delayed TNF activity to the pathology of inflammation has yet to be determined. Together, the release of HMGB1 by activated macrophages, its causative role in lethal endotoxemia, and its activity as a macrophage-stimulating agent (Table 1) reveal that HMGB1 is a late cytokine mediator of systemic inflammation.

PERSPECTIVES

HMGB1 was first identified as a nuclear chromosomal protein that participates in maintenance of nucleosomal structure and stability, and regulation of gene expression by facilitating the binding of transcription factors to their cognate DNA sequences (42). It is now clear that HMGB1 is also a late cytokine mediator of endotoxemia released into the extracellular milieu. The chronology of events in elucidating the biological role of HMGB1 shares an interesting antiparallel relationship with the discovery of IL-1α. IL-1α was initially characterized as a cytokine, but subsequently found to function as a transcription factor once transported from the cell membrane to the nucleus (78–80). It is interesting to consider that extracellular HMGB1 may be transported to the nucleus, where it might interact with transcription factors. The discovery of HMGB1 as a monocyte-derived, late-acting cytokine mediator of endotoxin lethality has initiated a new field of investigation for development of experimental therapeutics. The downstream or "late" action of HMGB1 is a marked departure from the early activities of TNF and other classic proinflammatory cytokines, and has significant implications for understanding and manipulating innate immune responses. Unpublished data (H. Wang and K. J. Tracey) suggest that it may be possible to develop antiinflammatory therapeutics that inhibit HMGB1 release and prevent death in murine sepsis. The insights gained from these and other studies of HMGB1 may widen the therapeutic window for treatment of endotoxemia, septic shock, and sepsis, if this newly recognized cytokine proves to be a clinically accessible target.

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